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(54) Title: PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

(57) Abstract

The tomato *Cf-9* gene has been cloned and its sequence provided, along with the encoded amino acid sequence. DNA encoding the polypeptide, alleles, mutants and derivatives thereof, and DNA encoding amino acid sequences showing a significant degree of homology thereto may be introduced into plant cells and the encoded polypeptide expressed, conferring pathogen resistance on plants comprising such cells and descendants thereof. The CF-9 sequence comprises leucine rich repeats and the presence of such repeats enables identification of other plant pathogen resistance genes.

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PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

The present invention relates to pathogen resistance in plants and more particularly the identification and use of pathogen resistance genes.

5   Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, because they are usually grown as genetically uniform monocultures; when disease strikes, losses can be severe. However, most plants are resistant to most

10   plant pathogens. To defend themselves, plants have evolved an array of both preexisting and inducible defences. Pathogens must specialize to circumvent the defence mechanisms of the host, especially those biotrophic pathogens that derive their nutrition from an

15   intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to be compatible, but if the plant is resistant, the interaction is said to be incompatible. Race specific resistance is strongly correlated with the hypersensitive

20   response (HR), an induced response by which (it is hypothesized) the plant deprives the pathogen of living host cells by localized cell death at sites of attempted pathogen ingress.

It has long been known that HR-associated disease

25   resistance is often (though not exclusively) specified by dominant genes (R genes). Flor showed that when pathogens mutate to overcome such R genes, these mutations are recessive. Flor concluded that for R genes

to function, there must also be corresponding genes in the pathogen, denoted avirulence genes (*Avr* genes). To become virulent, pathogens must thus stop making a product that activates *R* gene-dependent defence

5   mechanisms (Flor, 1971). A broadly accepted working hypothesis, often termed the elicitor/ receptor model, is that *R* genes encode products that enable plants to detect the presence of pathogens, provided said pathogens carry the corresponding *Avr* gene (Gabriel and

10   Rolfe, 1990). This recognition is then transduced into the activation of a defence response.

Some interactions exhibit different genetic properties. *Helminthosporium carbonum* races that express a toxin (Hc toxin) infect maize lines that lack the *Hm1*

.15   resistance gene. Mutations to loss of Hc toxin expression are recessive, and correlated with loss of virulence, in contrast to gene-for-gene interactions in which mutations <u>to</u> virulence are recessive. A major accomplishment was reported in 1992, with the isolation

20   by tagging of the *Hm1* gene (Johal and Briggs, 1992). Plausible arguments have been made for how gene-for-gene interactions could evolve from toxin-dependent virulence. For example, plant genes whose products were the target of the toxin might mutate to confer even greater

25   sensitivity to the toxin, leading to HR, and the conversion of a sensitivity gene to a resistance gene. However, this does not seem to be the mode of action of *Hm1*, whose gene product inactivates Hc toxin.

Pathogen avirulence genes are still poorly understood. Several bacterial *Avr* genes encode hydrophilic proteins with no homology to other classes of protein, while others carry repeating units whose number

5   can be modified to change the range of plants on which they exhibit avirulence (Keen, 1992; Long and Staskawicz, 1993). Additional bacterial genes (*hrp* genes) are required for bacterial *Avr* genes to induce HR, and also for pathogenicity (Keen, 1992; Long and Staskawicz,

10 1993). It is not clear why pathogens make products that enable the plant to detect them. It is widely believed that certain easily discarded *Avr* genes contribute to but are not required for pathogenicity, whereas other *Avr* genes are less dispensable (Keen, 1992; Long and

15 Staskawicz, 1993). The characterization of one fungal avirulence gene has also been reported; the *Avr9* gene of *Cladosporium fulvum*, which confers avirulence on *C. fulvum* races that attempt to attack tomato varieties that carry the *Cf-9* gene, encodes a secreted cysteine-rich

20 peptide with a final processed size of 28 amino acids but its role in compatible interactions is not clear (De Wit, 1992).

The technology for gene isolation based primarily on genetic criteria has improved dramatically in recent

25 years, and many workers are currently attempting to clone a variety of *R* genes. Targets include (amongst others) rust resistance genes in maize, *Antirrhinum* and flax (by transposon tagging); downy mildew resistance genes in

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lettuce and *Arabidopsis* (by map based cloning and T-DNA

tagging); *Cladosporium fulvum* (*Cf*) resistance genes in

tomato (by tagging, map based cloning and affinity

labelling with avirulence gene products); virus

5   resistance genes in tomato and tobacco (by map based

cloning and tagging); nematode resistance genes in tomato

(by map based cloning); and genes for resistance to

bacterial pathogens in *Arabidopsis* and tomato (by map

based cloning).

10   The map based cloning of the tomato *Pto* gene that

confers "gene-for-gene" resistance to the bacterial speck

pathogen *Pseudomonas syringae* pv tomato (Pst) has been

reported (Martin *et al*, 1993). A YAC (yeast artificial

chromosome) clone was identified that carried restriction

15   fragment length polymorphism (RFLP) markers that were

very tightly linked to the gene. This YAC was used to

isolate homologous cDNA clones. Two of these cDNAs were

fused to a strong promoter, and after transformation of a

disease sensitive tomato variety, one of these gene

20   fusions was shown to confer resistance to Pst strains

that carry the corresponding avirulence gene, *AvrPto*.

These two cDNAs show homology to each other. Indeed, the

Pto cDNA probe reveals a small gene family of at least

six members, 5 of which can be found on the YAC from

25   which *Pto* was isolated, and which thus comprise exactly

the kind of local multigene family inferred from genetic

analysis of other R gene loci.

5

The *Pto* gene cDNA sequence is puzzling for proponents of
the simple elicitor/receptor model. It reveals
unambiguous homology to serine/threonine kinases,
consistent with a role in signal transduction

5   Intriguingly, there is strong homology to the kinases
associated with self incompatibility in Brassicas, which
carry out an analogous role, in that they are required to·
prevent the growth of genotypically defined incompatible
pollen tubes. However, in contrast to the Brassica SRK

10   kinase (Stein *et al* 1991), the *Pto* gene appears to code
for little more than the kinase catalytic domain and a
potential N-terminal myristoylation site that could
promote association with membranes. It would be
surprising if such a gene product could act alone to

15   accomplish the specific recognition required to initiate
the defence response only when the *AvrPto* gene is
detected in invading microrganisms. The race-specific
elicitor molecule made by Pst strains that carry *AvrPto*
is still unknown and needs to be characterized before

20   possible recognition of this molecule by the Pto gene
product can be investigated.

We have now isolated the tomato *Cf-9* gene which
confers resistance against the fungus *Cladosporium fulvum*
and we have sequenced the DNA and deduced the amino acid

25   sequence from this gene. The DNA sequence of the tomato
Cf-9 genomic gene is shown in SEQ ID NO.1 (and Figure 2)
and the deduced amino acid sequence is shown in SEQ ID
NO. 2 (and Figure 3). A cDNA sequence is shown in SEQ ID

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NO. 4 (and Figure 4).

As described in more detail below, the tomato *Cf-9* gene was isolated by a method which involved use of a transformed line of tomato engineered for expression of

5   the *Avr9* avirulence gene. This transformed line, which constitutively expressed functional, mature Avr9 protein, was crossed to plants which carried the *Cf-9* gene so that a proportion of the progeny exhibited a necrotic phenotype culminating in seedling death. The *Cf-9* gene

10   was identified by the technique of transposon tagging with tagging of the *Cf-9* gene being confirmed by survival of the resulting seedlings.

According to one aspect, the present invention provides a DNA isolate encoding a pathogen resistance

15   gene or a fragment thereof, the gene being characterized in that it encodes the amino acid sequence shown in SEQ ID NO 2 or an amino acid sequence showing a significant degree of homology thereto.

For example, the DNA isolate comprises DNA encoding

20   an amino acid sequence showing 60% homology, preferably 80% homology, more preferably 90% homology to the amino acid sequence shown in SEQ ID NO 2. Most preferably the DNA encodes the amino acid sequence shown in SEQ ID NO 2 in which case the DNA isolate may comprise DNA having the

25   sequence shown in SEQ ID NO 1 or SEQ ID NO 4, or part of either of these sufficient to encode the desired polypeptide (eg from the initiating methionine codon to the first in frame downstream stop codon). In one

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embodiment the DNA comprises a sequence of nucleotides
which are the nucleotides 1871 to 2969 of SEQ ID NO 1, or
a mutant, derivative or allele thereof. A further aspect
of the invention provides a DNA isolate encoding a
5   pathogen resistance gene, or a fragment thereof,
obtainable by screening a DNA library with a probe
comprising nucleotides 1871 to 2969 of SEQ ID NO 1, or a
fragment, derivative, mutant or allele thereof, and
isolating DNA which encodes a polypeptide able to confer
10   pathogen resistance to a plant, such as resistance to
Cladosporium fulvum (eg. expressing Avr9). The plant may
be tomato. Suitable techniques are well known in the
art.

DNA according to the present invention may encode
15   the amino acid sequence shown in SEQ ID NO 2 or a mutant,
derivative or allele of the sequence provided. Preferred
mutants, derivatives and alleles are those which retain a
functional characteristic of the protein encoded by the
wild-type gene, especially the ability to confer pathogen
20   resistance. Changes to a sequence, to produce a mutant
or derivative, may be by one or more of insertion,
deletion or substitution of one or more nucleotides in
the nucleic acid, leading to the insertion, deletion or
subsitution of one or more amino acids. Of course,
25   changes to the nucleic acid which make no difference to
the encoded amino acid sequence are included.

The DNA isolate, which may contain the DNA encoding
the amino acid sequence of SEQ ID NO 2 or an amino acid

sequence showing a significant degree of homology thereto as genomic DNA or cDNA, may be in the form of a recombinant vector, for example a phage or cosmid vector. The DNA may be under the control of an appropriate

5 promoter and regulatory elements for expression in a host cell, for example a· plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory

10 elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences,

15 including promoter sequences, terminator fragments, polyadenylation seuqences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for· example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring

20 Harbor Laboratory Press.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct

25 which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into

the endogenous chromosomal material may or may not occur according to different embodiments of the invention. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into

5   whole plants.

Plants transformed with the DNA segment containing pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any

10   suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616)

15   microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous

20   species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective.

25   Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg. bombardment with Agrobacterium coated microparticles (EP-A-486234) or

mircoprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to
5   transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into
10   plant cells is not essential to or a limitation of the invention.

The *Cf-9* gene and modified versions thereof encoding a protein showing a significant degree of homology to the protein product of the *Cf-9* gene,
15   alleles, mutants and derivatives thereof, may be used to confer resistance in plants, in particular tomatoes, to a pathogen such as *C. fulvum*. For this purpose a vector as described above may be used for the production of a transgenic plant. Such a plant may possess pathogen
20   resistance conferred by the *Cf-9* gene.

The invention thus further encompasses a host cell transformed with such a vector, especially a plant or a microbial cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present
25   invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome.

A vector comprising nucleic acid according to the present invention need not include a promoter,

particularly if the vector is to be used to introduce the
nucleic acid into cells for recombination into the
genome.

Also according to the invention there is provided a
plant cell having incorporated into its genome a sequence
of nucleotides as provided by the present invention,
under operative control of a promoter for control of
expression of the encoded polypeptide. A further aspect
of the present invention provides a method of making such
a plant cell involving introduction of a vector
comprising the sequence of nucleotides into a plant cell.
Such introduction may be followed by recombination
between the vector and the plant cell genome to introduce
the sequence of nucleotides into the genome. The
polypeptide encoded by the introduced nucleic acid may
then be expressed.

Plants which comprise a plant cell according to the
invention are also provided, along with any part or clone
thereof, seed, selfed or hybrid progeny and descendants.

The invention further provides a method of
comprising expression from nucleic acid encoding the
amino acid sequence SEQ ID NO 2, or a mutant, allele or
derivative thereof, or a significantly homologous amino
acid sequence, within cells of a plant (thereby producing
the encoded polypeptide), following an earlier step of
introduction of the nucleic acid into a cell of the plant
or an ancestor thereof. Such a method may confer
pathogen resistance on the plant.

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A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, cells of which decendants may express the encoded polypeptide and so may have enhanced pathogen resistance. Pathogen resistance may be determined by assessing compatibility of a pathogen (eg. *Cladosporium fulvum* or using recombinant expression of a pathogen avirulence gene, such as *Avr9*. Such a gene may be introduced into cells of a plant by any suitable transformation technique or by cross-breeding, as discussed herein.

Sequencing of the *Cf-9* gene has shown that it includes DNA sequence encoding leucine-rich repeat (LRR) regions and homology searching has revealed strong homologies to other genes containing LRRs. For the reasons discussed in more detail below, the presence of LRRs can be hypothesised to be characteristic of many pathogen resistance genes and the presence of LRRs can thus be used in a method of identifying further pathogen resistance genes.

According to a further aspect, the present invention provides a method of identifying a plant pathogen resistance gene which comprises:

(1)   obtaining expressed or genomic DNA from cells of a plant possessing resistance to a pathogen;

(2)   sequencing the DNA and identifying putative pathogen resistance genes by the presence of LRRs;

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and

(3)   confirming identification as a pathogen resistance gene.

DNA which may contain a pathogen resistance gene
5   can be obtained in many ways.   In the course of map-based
cloning of disease resistance genes, genetic analysis may
identify YAC clones that may possibly carry the
resistance gene.   Such YAC clones could then be used to
screen cDNA clones from a cDNA library, and homologous
10   cDNA clones that mapped from the region sequenced.   These
sequences can then be inspected for the presence of LRRs
and putative pathogen resistance genes identified on the
basis of such LRRs.

Alternatively, random DNA sequences from an
15   appropriate plant source can be obtained, for example as
cDNA or as genomic DNA in a cosmid vector or YAC, and
this random DNA can be sequenced and putative pathogen
resistance genes identified on the basis of LRRs.   A
large amount of DNA sequence information has already been
20   generated from DNA derived from many different sources
and this sequence information is available in databases.
Such known DNA sequences can be searched for LRRs and
sequence from an appropriate source showing LRRs can
again be identified as a putative pathogen resistance
25   gene.

LRRs are already known in many different genes (see
for example Chang et al 1992) so that sequences of this
type can readily be identified.   Identification of LRRs

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can be by simple visual inspection of the sequence to
find areas of sequence that carry repeated motifs that
are rich in leucine residues. Alternatively an
appropriate computer searching technique can be used to

5   determine homology to a known sequence containing LRRs or
to a consensus sequence derived from known sequences
containing LRRs. More particularly, use can be made of
one or other of the various available algorithms for
local sequence similarity searching such as BLASTX.

10   Thus, for example, a BLASTX search can be used in
databases at the US National Center for Biological
Information and an LRR containing sequence can be
identified by a BLASTX score of at least 60 or more
against the sequence for *Cf-9* as set out in SEQ ID NO 2.

15   Once a putative pathogen resistance gene has been
identified, this can be investigated further, if
necessary following isolation of the full coding
sequence, by linkage analysis to determine the chromosome
on which the gene is located and whether it is linked to

20   known locations for pathogen resistance genes. Such
linkage analysis may also give indications as to the
nature of the pathogen involved. Following linkage
analysis, identification of a pathogen resistance gene
can be confirmed by reintroduction of the DNA back into a

25   plant with an appropriate genotype and investigation of
the effect of that DNA on the transformed plant. If the
effect is to confer resistance to a specific pathogen to
an otherwise non-resistant plant, then this confirms the

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gene as a pathogen resistance gene.

The techniques described above are of general applicability to the identification of pathogen resistance genes in plants. Examples of the type of

5   genes that can be identified in this way include *Phytophthora* resistance in potatoes, mildew resistance and rust resistance in cereals such as barley and maize, rust resistance in *Antirrhinum* and flax, downy mildew resistance in lettuce and *Arabidopsis*, virus resistance

10   in potato, tomato and tobacco, nematode resistance in tomato, resistance to bacterial pathogens in *Arabidopsis* and tomato and *Xanthomonas* resistance in peppers.

Once a pathogen resistance gene has been identified, it can be reintroduced into the plant in

15   question by techniques well known to those skilled in the. art to produce transgenic plants that have been engineered to carry the resistance gene in question. According to a further aspect, the present invention provides a DNA isolate encoding the protein product of a

20   plant pathogen resistance gene which has been identified by use of the presence therein of LRRs and, in particular, by the technique defined above. According to a yet further aspect, the invention provides transgenic plants, in particular crop plants, which have been

25   engineered to carry pathogen resistance genes which have been identified by the presence of LRRs. Examples of suitable plants include tobacco, cucurbits, carrot, vegetable brassica, lettuce, strawberry, oilseed

brassica, sugar beet, wheat, barley, maize, rice, soyabeans, peas, sorghum, sunflower, tomato, potato, pepper, chrysanthemum, carnation, poplar, eucalyptus and pine.

5   Further aspects and embodiments of the patent invention will be apparent to those skilled in the art. All documents mentioned herein are incorporated by reference.

As already indicated, the present invention is
10 based on the cloning and sequencing of the tomato *Cf-9* gene and this experimental work is described in more detail below with reference to the following figures.

Figure 1 shows a schematic representation of the
15 *Cf-9* gene.

Figure 2 shows the genomic DNA sequence of the *Cf-9* gene (SEQ ID NO 1). Features: Nucleic acid sequence - Translation start at nucleotide 898; translation stop at nucleotide 3487; polyadenylation signal (AATAAA) at
20 nucleotide 3703-3708; polyadenylation site at nucleotide 3823; a 115 bp intron in the 3' non-coding sequence from nucleotide 3507/9 to nucleotide 3622/4. Predicted Protein Sequence - primary translation product 863 amino acids; signal peptide sequence amino acids 1-23; mature
25 peptide amino acids 24-863.

Figure 3 shows Cf-9 protein amino acid sequence (SEQ ID NO 2).

Figure 4 shows the sequence of one of the CF9 cDNA

clones (SEQ ID NO 4). Translation initiates at the ATG
at position +58.

Figure 5 shows a physical map of the tomato *Cf-9*
locus generated from overlapping cosmids (34, 41, 110 and
5   138) isolated from the *Cf-2/Cf-9* cosmid library. The
extent of each cosmid and location of the Cf-9 gene are
shown schematically. Also indicated are the direction of
the transcription (arrow) and the location of sites for
restriction enzyme *Bgl*II (B).

10

Cloning of the tomato Cf-9 gene

As already indicated, the *C. fulvum* AVR9 gene and
product are known (De Wit, 1992; van Kan et al 1991;
Marmeisse *et al* 1993; Van Den Ackerveken *et al* 1993),
15   Accordingly isolation of the *Cf-9* gene would be
scientifically attractive, because it should enable
characterization of binding between the AVR9 gene product
ligand and the presumed *Cf-9* gene product receptor.

20   **(i) Assignment of *Cf-* gene map locations.**

We have mapped several *Cf* genes, including *Cf-9*, to
their chromosomal locations (Dickinson et al 1993; Jones
et al 1993; Balint-Kurti *et al* 1993). We showed that
Cf-4 and *Cf-9* map to approximately the same location on
25   the short arm of chromosome 1, and *Cf-2* and *Cf-5* map to
approximately the same location on chromosome 6. Others
independently mapped *Cf-9* to chromosome 1 (van der Beek
et al 1992).

(ii) Establishing transposon tagging in tomato

We have been establishing the capacity to carry out transposon tagging in tomato using the maize transposon *Activator* (*Ac*) and its *Dissociation* (*Ds*) derivatives (Scofield *et al* 1992; Thomas *et al* 1994; Carroll *et al* 1995). The strategy is founded on the fact that these transposons preferentially transpose to linked sites. Accordingly we have made available lines that carry *Ds*s at positions that are useful to our colleagues. J Hille made available a line, FT33 (Rommens *et al* 1992), carrying a *Ds* linked to *Cf-9*. We have independently generated our own lines that carry a construct SLJ10512 (Scofield et al 1992) which contains (a) a beta-glucuronidase (*GUS*) gene (Jefferson *et al* 1987) to monitor T-DNA segregation and (b) stable *Ac* (*sAc*) that expresses transposase and can trans-activate a Ds, but which will not transpose (Scofield *et al* 1992).

(iii) Establishing a stock from which gametes carrying a mutagenized *Cf-9* gene could be obtained and identified

The line FT33 did not carry a *Cf-9* gene. We had to obtain recombinants that placed *Cf-9* *in cis* with the T-DNA in FT33 in order to carry out linked targeted tagging. Two strategies were pursued simultaneously.

(a) FT33 was crossed to Cf9, a stock that carries the *Cf-9* gene. The resulting F1 was then back crossed to Cf0 (a stock that carries no *Cf-* genes). Progeny that carry the FT33 T-DNA are kanamycin resistant. Kanamycin

resistant progeny were tested for the presence of *Cf-9*;
5 *C. fulvum* resistant individuals were obtained among
180.   We also generated progeny that were homozygous for
Cf-9 and carried the s*Ac* T-DNA of SLJ10512.   These were
5   crossed to the recombinants in which *Cf-9* and FT33 were
in cis.   In the FT33 T-DNA, a transposable *Ds* element is
cloned into a hygromycin resistance gene, preventing its
function.   The somatic transactivation of this *Ds*
element,   which only occurs in the presence of
10   transposase gene expression, results in activation of the
hygromycin resistance.   Thus from crossing the
recombinants between *Cf-9* and FT33,   to the s*Ac*-carrying
Cf-9 homozygotes, hygromycin resistant individuals could
be obtained which carry s*Ac* and FT33,   and are likely to
15   be homozygous for *Cf-9*.   140 individuals of this genotype
were thus obtained.

(b)   To accelerate obtaining individuals that
carried s*Ac*, FT33, and were *Cf-9* homozygotes,   the
FT33/*Cf-9* F1 was crossed to a line that was heterozygous
20   for *Cf-9* and s*Ac*.   25% of the resulting progeny carried
both T-DNAs and were hygromycin resistant, and of those,
slightly more than 50% were disease resistant because
they carried at least one copy of the *Cf-9* gene.   A
restriction fragment length polymorphism (RFLP) marker
25   was available, designated CP46,   that enabled us to
distinguish between homozygotes and heterozygotes for the
Cf-9 gene (Balint-Kurti *et al* 1994 (in press)).   In this
manner two individuals that were *Cf-9* homozygotes,   and

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that carried both the FT33 T-DNA and s*Ac*, were obtained.
These two individuals were multiplied by taking cuttings
so that more crosses could be made onto this genotype.

5   **(iv) Establishing a tomato stock that expresses
       functional mature AVR9 protein**

       A likely frequency for obtaining any desired
mutation in a gene tagging experiment is less than 1 in
1000, and often less than 1 in 10,000 (Döring, 1989).

10   To avoid screening many thousands of plants for mutations
to disease sensitivity, we established a selection for
such mutations based on expressing the fungal *Avr9* gene
in plants. The sequence of the 28 amino acids of the
mature Avr9 protein is known (van Kan *et al* 1991). It is

15   a secreted protein and can be extracted from
intercellular fluid of leaves infected with Avr9-carrying
races of *C. fulvum*. For secretion from plant cells, we
designed oligonucleotides to assemble a gene that carried
a 30 amino acid plant signal peptide, from the Pr1a gene

20   (Cornelissen *et al* 1987) preceding the first amino acid
of the mature Avr9 protein (see SEQ ID No. 3). The
preferred *Avr9* gene sequence depicted in SEQ ID No. 3 is
a chimaeric gene engineered from the Pr-1a signal peptide
sequence (Cornelissen *et al* 1987) and the *Avr9* gene

25   sequence (van Kan *et al*, 1991). This reading frame was
fused to the 35S promoter of cauliflower mosaic virus
(Odell *et al* 1984), and the 3' terminator sequences of
the octopine synthase gene (DeGreve *et al* 1983), and

introduced into binary plasmid vectors for plant transformation, using techniques well known to those skilled in the art, and readily available plasmids (Jones *et al* 1992). We obtained transformed Cf0 tomato

5   lines that expressed this gene. These transformed lines were crossed to plants that carried the *Cf-9* gene. When the resulting progeny were germinated, 50% exhibited a necrotic phenotype, that culminated in seedling death. This outcome was only observed in seedlings that

10   contained the *Avr9* gene. When the same transformants were crossed to Cf0 plants, the resulting prgeny were all fully viable. From selfing the primary transformants, individuals were identified that were homozygous for the *Avr9* transgene. When *Avr9* homozygotes

15   were crossed to *Cf-9*, all progeny died. This system thus provides a powerful selection for individuals that carry mutations in the *Cf-9* gene (Hammond-Kosack et al 1994).

(v) Tagging the *Cf-9* gene

20   Individuals that were homozygous for the *Avr9* gene (section (iv)) were used as male parents to pollinate individuals that were homozogyous for *Cf-9*, and carried both s*Ac* and the *Ds* in the FT33 T-DNA (section (iiia) and (iiib)). Many thousands of progeny resulting from such a

25   cross were germinated. Most died, but some survived.

       DNA was obtained from survivors and subjected to Southern blot analysis using a *Ds* probe. It was observed that several independent mutations were correlated with

22

insertions of the *Ds* into a BglII fragment of a

consistent size. The same result was observed with XbaI.

This sugested that several independent mutations were a

consequence of insertion of the *Ds* into the same DNA

5   fragment.

Using primers to the *Ds* sequence, DNA adjacent to

the *Ds* in transposed *Ds*-carrying mutant #18 was amplified

using inverse PCR (Triglia *et al* 1988). This DNA was be

used as a probe to other mutants, and proved that in

10 independent mutations, the *Ds* had inserted into the same

6.7 kb BglII fragment.

The *Ds* in FT33 contains a bacterial replicon and a

chloramphenicol resistance gene as a bacterial selectable

marker (Rommens *et al* 1992). This means that plant DNA

15 carrying this transposed *Ds* can be digested with a

restriction enzyme that does not cut within the *Ds* (such

as BglII), the digestion products can be recircularized,

and then used to transform *E. coli*. Chloramphenicol

resistant clones can be obtained that carry the *Ds* and

20 adjacent plant DNA. This procedure was used to obtain a

clone that carried 1.7 kb of plant DNA on the 3' side of

the *Ds*, and 4.9 kb of plant DNA on the 5' side of the *Ds*.

Our current understanding of the *Cf-9* gene is

depicted schematically in Figure 1. The 1.8 kb of plant

25 DNA on the 3' side of Ds extend between insertion #18 and

the Bg1ll site on this figure. Further clones were

obtained by digesting plant DNA of mutant #18 with Xbal

instead of Bg1ll prior to recircularization and

23

transformation.   This permitted the isolation of clones
carrying DNA that extended considerably (at least 5kb) to
the right of this Bglll site, and thus permitted
sequencing of DNA to the right of the Bglll site shown in
5   Figure 1.

Using a combination of various subclones, synthesis
of new sequencing primers for further sequence
determination based on newly established sequence
(primers F1, 2, 3, 4, 5, 6, 7, 12, 13, 10, 26, 27 and 25
10   that were used in such experiments are indicated in the
Figure), and other techniques well known to those skilled
in the art, 3847 bp of sequence were determined.   Various
other restriction sites (Xhol, Sstl, EcoRI and Hindlll)
are also indicated in Figure 1.

15   The F-series of primers were used to characterise a
large number of independent mutations by PCR analysis in
combination with primers based on the sequence of Ds.
Therefore, these primers were used in polymerase chain
reactions with primers based on the maize Ac/Ds
20   transposon sequence, to characterise the locations of
other mutations of Cf-9 that were caused by transposon
insertion.

Eighteen independent insertions were characterized
and are located as shown.   Mutants E, #55, #74 and #100
25   gave incomplete survival and showed a necrotic phenotype,
and based on the available sequence information, they are
5' to the actual reading frame and might permit enough
Cf9 protein expression to activate an incomplete defence

24

response.

(vi) DNA sequence analysis of the *Cf-9* gene

DNA sequence analysis of the *Cf-9* gene has now been

5   completed and upon conceptual translation has revealed an

interesting motif (the leucine rich repeat, or LRR) that

can be hypothesized to be diagnostic of other resistance

genes. The genomic DNA sequence of *Cf-9* is shown in

Figure 2 and SEQ ID NO. 1. Approximately 3.9 kbp of

10   genomic DNA sequence has been determined. A translation

start codon (ATG) sequence is located at position 898 and

a translation termination codon TAG sequence is located

at position 3487 bp (Figure 2), with an intervening

uninterrupted 863 amino acid open reading frame.

15   Using the sequence obtained, oligonucleotide

primers were designed that could be used in PCR reactions

in combination with primers based on the sequence of the

Ds element, to characterize both the location and the

orientation of other transposon insertions in the gene.

20   Based on the results of such experiments, the map

positions of 17 other Ds insertions can now be reliably

assigned (as shown in Figure 1).

The fact that 18 independent mutants that survive

in the presence of Avr9, are associated with insertions

25   into the same region of DNA, provides compelling evidence

that the *Cf-9* gene has been tagged, and that DNA sequence

obtained from this region is derived from the *Cf-9* gene.

25

Further proof is provided by the fact that when
mutant # 18, (a stable mutant that lacks sAc) is
back-crossed to a line homozygous for sAc, one quarter of
the resulting progeny carry sAc, Ds # 18, and the Avr9

5   gene. These progeny exhibit variegation for a necrosis,
consistent with the idea that on sAc dependent somatic
excision of Ds, Cf-9 gene function is somatically
restored, leading to sectors that die.
Further proof is provided by the fact that individuals

10   that survived the Avr9 selection lost disease resistance
to races of C. fulvum that carry the Avr9 gene (Jones et
al. 1994).

(vii) Identification of a leucine-rich repeat region in

15   **Cf-9.**

The genomic DNA sequence of the Cf-9 gene is shown
in Figure 2 (SEQ ID NO. 1). The deduced amino acid
sequence of the Cf-9 protein is shown in Figure 3 (SEQ ID
NO. 2). Currently the 18 independent Ds insertions are

20   all in or 5' to the 863 amino acid open reading frame
shown in Figure 3. A cDNA library was constructed from
messenger RNA isolated from tomato cotyledons injected
with intercellular fluid containing AVR9 peptide in a
bacteriophage lambda cloning vector. 600,000 cDNA clones

25   were screened and 18 clones were identified that
hybridized to DNA probes from sequences adjacent to the
Ds insertions in the Cf-9 gene. While some of these cDNA
clones were from other members of the Cf-9 multigene

26

family (Jones et al 1994), six clones were identified
that are derived from the genomic sequence shown in
Figure 2 because they show identical DNA sequence apart
from the splicing out of a small intron in the 3'

5   untranslated region between nucleotides 3509 and 3623 of
the Figure 2 sequence. The sequence of one such cDNA
clone is shown below in Figure 4 (SEQ ID NO. 4).

Homology searching of the resulting sequence
against sequences in the databases at the US National

10 Centre of Biological Information (NCBI) reveals strong
homologies to other genes that contain leucine rich
repeat regions (LRRs). These include the Arabidopsis
genes TMK1 (Chang at al 1992), TMKL1 (Valon et al 1993),
RLK5 (Walker, 1993), as well as expressed sequences with

15 incomplete sequence and unknown function (e.g.
Arabidopsis thaliana transcribed sequence [ATTS] 1447).
The presence of LRRs has been observed in other genes,
many of which probably function as receptors (see Chang
et al (1992) for further references).

20   The TMK1 and RLK5 genes have structures which
suggest they encode transmembrane serine/threonine
kinases and carry extensive LRR regions. As yet no known
function has been assigned to them. Disease resistance
genes are known to encode gene products which recognize

25 pathogen products and subsequently initiate a signal
transduction chain leading to a defence response. It is
known that another characterized disease resistance gene
(Pto) is a protein kinase (Martin et al 1993). However,

in *Cf-9* there is no apparent protein kinase domain based on genomic DNA and cDNA sequence analysis.

5   The predicted Cf-9 amino acid sequence can be divided into 7 domains (see also figure 3 in Jones et al 1994):

Domain A is a 23 amino acid probable signal peptide.

Domain B is a 68 amino acid region with some homology to polygalacturonase inhibitor proteins.

10   Domain C is a 668 amino acid comprising 28 imperfect copies of a 24 amino acid leucine rich repeat (LRR).

Domain D is a 28 amino acid domain with some homology to polygalacturonase inhibitor proteins.

15   Domain E is a 18 amino acid domain rich in negatively charged residues.

Domain F is a 37 amino acid hydrophobic domain encoding a putative transmembrane domain.

Domain G is a 21 amino acid domain rich in
20   positively charged residues.

Domains E, F and G together comprise a likely membrane anchor.

(viii) Isolation of binary cosmid vector clones that
25   **carry a genomic *Cf-9* gene**

In order to demonstrate that the gene characterized by transposon tagging is indeed*Cf-9*, we have demonstrated that homologous DNA sequences from the Moneymaker Cf9

near isogenic line (the Cf9 stock) could confer both

resistance to *C. fulvum* and sensitivity to Avr9 peptide

in transgenic Cf0 tomato plants into which these

sequences have been transformed.

5   A genomic DNA library was constructed from a stock

that carried both the *Cf-9* gene on chromosome 1, and the

Cf-2 gene on chromosome 6, so that the library could be

used for isolating both genes. The library was

constructed in a binary cosmid cloning vector pCLD04541,

10   obtained from Dr C. Dean, John Innes Centre, Colney Lane,

Norwich (see also Bent et al., 1994). This vector is

essentially similar to pOCA18 (Olszewski et al., 1988).

It contains a bacteriophage lambda cos site to render the

vector packageable by lambda packaging extracts and is

15   thus a cosmid (Hohn and Collins, 1980). It is also a

binary vector (van den Elzen et al., 1985), so any cosmid

clones that are isolated can be introduced directly into

plants to test for the function of the cloned gene.

High molecular weight DNA was isolated from leaves

20   of 6 week old greenhouse-grown plants by techniques well

known to those skilled in that art (Thomas *et al* 1994)

and partially digested with MboI restriction enzyme. The

partial digestion products were size fractionated using a

sucrose gradient and DNA in the size range 20-25

25   kilobases (kb) was ligated to BamHI digested pCLD04541

DNA, using techniques well known to those skilled in the

art. After *in vitro* packaging using Stratagene packaging

extracts, the cosmids were introduced into a tetracycline

29

sensitive version (obtained from Stratagene) of the
Stratagene *Escherichia coli* strain SURE™. Recombinants
were selected using the tetracycline resistance gene on
pCLD04541.

5   The library was randomly distributed into 144 pools
containing about 1500 clones per pool, cells were grown
from each pool and from 10 ml of cells, 9 ml were used
for bulk plasmid DNA extractions, and 1 ml was used after
addition of 0.2 ml of glycerol, to prepare a frozen

10   stock. Plasmid DNA was isolated by alkaline lysis
(Birnboim and Doly, 1979), and was analyzed by PCR for
pools that might carry *Cf-9* homologous DNA, using the PCR
primers F7 and F10 with the sequences
5'GGAAGAGATGTTTACAGATTCAAGG3' (SEQ ID NO 5) and

15   5'ATCAGCAGGTCGATTCTTGTGG3' (SEQ ID NO 6) respectively,
that prime towards each other from positions 707-728 and
1494-1518 of the genomic DNA sequence. Pools 34, 41, 110
and 138 proved to be positive by this assay.

        For each pool, approximately 10,000 colonies were

20   plated out and inspected for *Cf-9* homology by colony
hybridization with a radioactive *Cf-9* probe, and from
each pool, single clones were isolated that carried such
homology and gave a PCR product upon carrying out a PCR
reaction with the F7, F10, combination of primers. These

25   techniques are all well known to those skilled in the
art.

        These clones have been further characterized by
Southern blot hybridization using a *Cf-9* probe, and by

restriction enzyme mapping. Our current assessment of the
extent of contiguous DNA around *Cf-9*, as defined by these
overlapping cosmids is shown in Figure 5. These cosmids
were subsequently used in plant transformation

5   experiments, selecting for plant cells transformed to
kanamycin resistance, using techniques well known to
those skilled in the art. Transgenic tomato, tobacco and
potato plants were produced (Fillatti et al., 1987;
Hammond-Kosack et al., 1994; Horsch et al., 1985,

10   Spychalla and Bevan, 1993) with at least one of each of
cosmids 34, 41, 110 and 138.

**(ix) Assessment of *Cf-9* function in transgenic tomato,
tobacco and potato**

15   The function of a putative cloned *Cf-9* gene can be
assessed in transformed tomato by testing transformants
not only for resistance to Avr9-carrying *C. fulvum*, but
also for a necrotic response to intercellular fluid (IF)
containing active Avr9 peptide. The function of a cloned

20   *Cf-9* gene in species that are not a host for *C. fulvum* ,
such as tobacco and potato, can only be assessed by
evaluating the response to IF.

          To assess the biological activity conferred upon
tomato, potato and tobacco primary transformants carrying

25   different *Cf-9* cosmids, the interveinal panels of mature
leaves were injected with IFs either containing or
lacking Avr9 peptide. These IFs were prepared according
to the procedure of de Wit and Spikman (1982). The IFs

containing Avr9 peptide were obtained from either a compatible *C. fulvum* interaction involving race 0 and Cf0 plants or transgenic tobacco plants homozygous for the 35S:Avr9 construct (SLJ 6201) (Hammond-Kosack et al.

5   1994). The IFs lacking Avr9 were obtained from either a compatible *C. fulvum* interaction involving race 2,4,5,9 and Cf0 plants or from untransformed tobacco plants.

A summary of the results from experiments with the various cosmids introduced into tomato, tobacco and

10   potato is shown in Table 1. All the tomato plants that carried a functional *Cf-9* gene by the criterion of Avr9-induced necrosis, were also resistant to infection by *C. fulvum* races that express Avr9, unlike the *C. fulvum*- sensitive Cf0 Moneymaker variety into which the

15   cosmid clone had not been transformed.

A *Cf-9 - Avr9* - dependent grey necrotic response occurred within the IF injected leaf panels of most tomato (17 out of 23), potato (5 out of 5) and tobacco (10 out of 13) transformants by 24 hours post injection.

20   These data indicate that the genomic *Cf-9* gene, under the control of its own promoter, is functional and exhibits the expected specificity of action when introduced into various plant species, including tomato, potato and tobacco.

25   Further confirmation of the biological activity of *Cf-9* in tobacco was obtained by crossing 5 different primary transformants carrying a single copy of cosmid 34 (transformed lines B, H, I, L and M), to transgenic

32

tobacco plants homozygous for the 35S:Avr9 T-DNA.

Seedling lethality occurred in 50% of the F_1 progeny by 11

days after seed planting. A similar seedling lethal

phenotype was obtained when tomato plants carrying *Cf-9*

5   were crossed to 35S:Avr9 expressing tomato plants

(Hammond-Kosack et al. 1994). These data demonstrate the

feasibility of strategies that exploit the recognition

between Avr9 and Cf-9 for engineering disease resistance

in transgenic plants other than tomato.

TABLE 1

Plant Species	Trans'd Line	Cos'd #34	Cos'd #41	Cosmid #110	Cosmid #138
Tomato	A	+	+	+	+
	B	+	+	+	+
	C	−	+	+	+
	D	−	−	+	+
	E	−	+	−	+
	F	−	+		+
Potato	A			+	+
	B	+		+	+
Tobacco	A	−			
	B[1]	+[2]			
	C	−			
	D	+			
	E	+			
	F	+			
	G[1]	−			
	H[1]	+			
	I	+			
	J	+			
	K	+			
	L[1]	+			
	M[1]	+			

The response of transgenic tomato, potato and tobacco plants (primary transformants) carrying different *Cf-9* cosmid constructs to intercellular fluid containing Avr-9

34

peptide obtained from transgenic tobacco plants

homozygous for the 35S::Avr9 constructs (SLJ 6201). A

plus (+) indicates that grey necrotic symptoms formed

within the injected leaf panel by 24hrs. A minus (-)

5   indicates that there was no response. Copy numbers of

cosmid inserts were determined by Southern blot analysis.

¹   Single copy of cosmid 34, used for crossing with

10   transgenic tobacco plants homozygous for the

35S::Avr9 T-DNA.

²   Plants also respond positively to IF containing

Avr9 peptide obtained from a compatible *C.fulvum*

interaction (race 0 - Cf0) but give no response to

15   two different intercellular fluids lacking Avr9

(race 2,4,5,9 - Cf0) and untransformed tobacco.

KEY TO FIGURE 1

Figure 1 shows tagged alleles of the *Cf-9* gene. X

20   is a probable promoter.

SEQ ID NO 3.

The amino acid sequence and DNA sequence of the preferred form of the chimaeric *Avr9* gene used as described herein.

5   ATG GGA TTT GTT CTC TTT TGA CAA TTG CCT TCA TTT CTT CTT GTC

      M   G   F   V   L   F   S   Q   L   P   S   F   L   L   V

      TCT ACA CTT CTC TTA TTC CTA GTA ATA TCC CAC TCT TGC CGT GCC

      S   T   L   L   L   F   L   V   I   S   H   S   C   R   A

10

      TAC TGT AAC AGT TCT TGT ACA AGA GCT TTT GAC TGT CTT GGA CAA

      Y   C   N   S   S   C   T   R   A   F   D   C   L   G   Q

      TGT GGA AGA TGC GAC TTT CAT AAG CTT CAA TGT GTA CAT TGA

15   C   G   R   C   D   F   H   K   L   Q   C   V   H

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Claims:

1.   A DNA isolate encoding a pathogen resistance gene or fragment thereof, the gene being characterised in that it encodes the amino acid sequence shown in SEQ ID NO 2

5   or an amino acid sequence showing a significant degree of homology thereto.

2.   A DNA isolate as claimed in claim 1 which encodes an amino acid sequence as shown in SEQ ID NO 2 or an

10   allele, mutant or derivative thereof.

3.   A DNA isolate as claimed in claim 2 which comprises DNA having the sequence shown in SEQ ID NO 1 or SEQ ID NO 4.

15

4.   A recombinant vector in which DNA as claimed in any one of claims 1 to 3 is under control of an appropriate promoter and regulatory elements for expression in a host cell.

20

5.   Use of a DNA isolate according to any one of claims 1 to 3 or a recombinant vector according to claim 4 for the production of a transgenic plant.

45

6.   A host cell comprising a DNA isolate according to any one claims 1 to 3 or a recombinant vector according to claim 4.

5   7.   A host cell according to claim 6 which is a microbial cell.

8.   A host cell according to claim 6 which is a plant cell.

10

9.   A plant or any part thereof comprising a plant cell according to claim 8.

10.   Seed, selfed or hybrid progeny or a descendant of a

15   plant according to claim 9, or any part thereof.

11.   A method of conferring pathogen resistance on a plant, comprising expression from nucleic acid encoding the amino acid sequence shown in SEQ ID NO 2, or a

20   mutant, allele or derivative thereof or a significantly homologous amino acid sequence, within cells of the plant, following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

12.   A method according to claim 11 wherein the nucleic

acid comprises the sequence shown in SEQ ID NO 1 or SEQ

ID NO 4.

5   13.   A method of identifying a plant pathogen resistance

gene which comprises:

        (1)   obtaining expressed or genomic DNA from

        cells of a plant possessing resistance to a

        pathogen;

10   (2)   sequencing the DNA and identifying putative

        pathogen resistance genes by the presence of

        leucine rich repeats (LRRs), and

        (3)   confirming identification as a pathogen

        resistance gene.

15

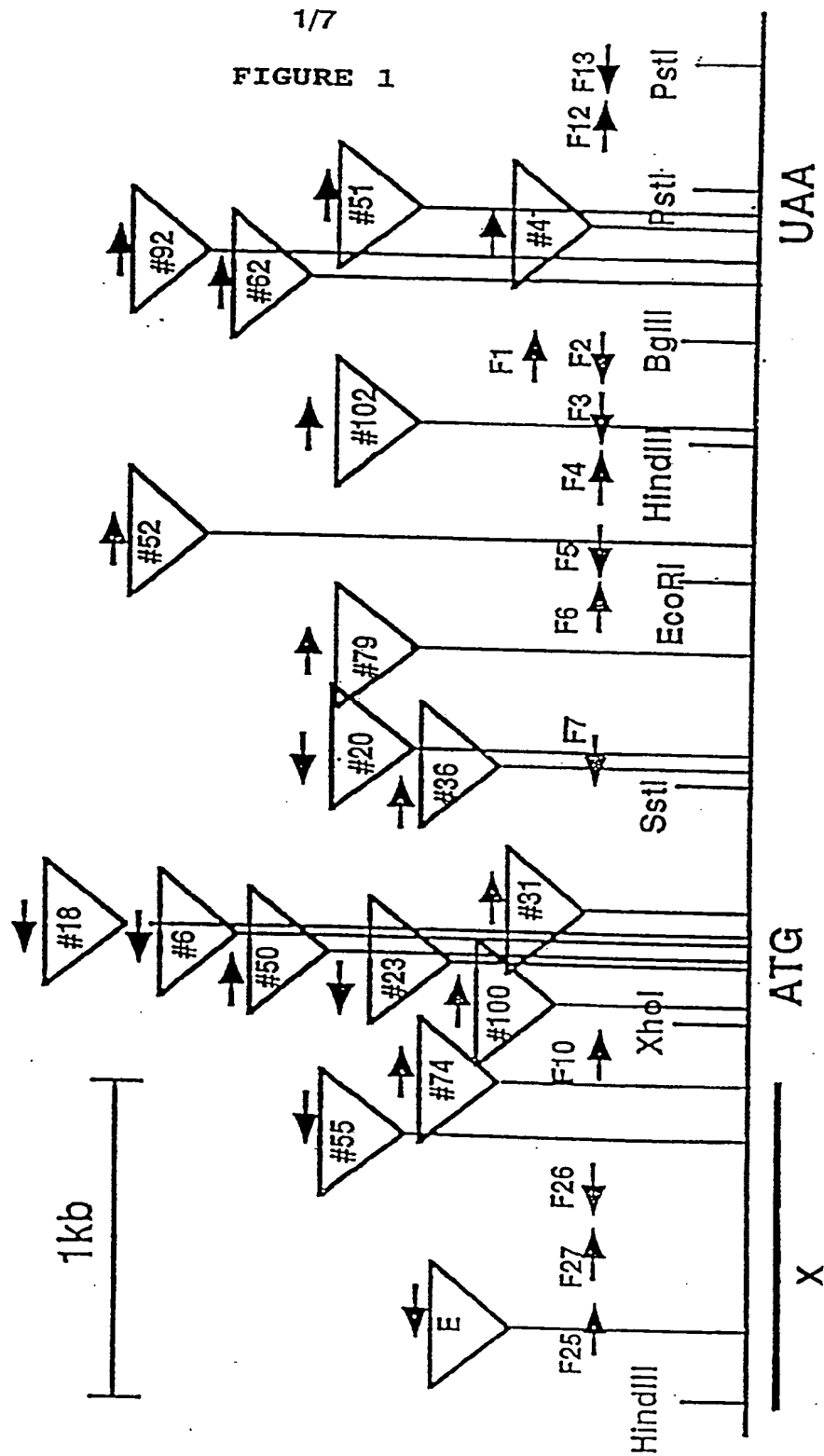14.   A method as claimed in claim 13 wherein LRRs are

identified as having a BLASTX score of 60 or more when

compared to the sequence of SEQ ID NO. 2.

20   15.   A method according to claim 13 or claim 14 wherein

identification as a pathogen resistance gene is confirmed

by linkage analysis and/or the effect of the gene on the

phenotype of an appropriate plant transformed therewith.

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FIGURE 1

FIGURE 2

```
   1   CATAGTCTTT GCATATTTGG ATTAAACAGG GGCATTATTG AACCAAACTA
  51   TTAGATGTAT GAAAATTTTG GACCAAGCTA TTGACAACAC GAACATTTTT
 101   AGACCAAACT ATTAACTCAG AATATTTTCC GTTGAATGAA TAAGGTAACT
 151   AGTAGTAAAT TTTTAGACCA AACTATGAAG AACATGCCAT GTCTGGACTC
 201   CTGCACTATC TTCCATCAAC AGGTCAATTC TCTCAACTCT ATTGGTGGAA
 251   GGTAGACGGT ACAAATTGAA TTATATTAAA AGACAAGCTC ACCTGAGCAT
 301   CACTGTTATA CAACAACAAC AAACTACGCT TCAGCCCCAA ACAATAGTGA
 351   CCCGAATCAT ATATTGTCAC GAGTTTTTTT TAGAGTATGT TGCATATATT
 401   ATACTCAACT TAGGGTTTGT CATTCTGATG CTTCGTACAA ATTTATTGAA
 451   TTTTCAACTT TAAAGGTTTA TGAACCAAAT ATTACGCTTA CTATGATAGC
 501   GGTCTTTTTT GATTAATCAA ACTTATTGAA TTTTCAACTT TAAAGGTTTT
 551   TCCCCGTTCT ATACACAAAC TAAGAAAAAT TTAAATTATA TAGTCTTTGG
 601   ATGGTGACCT ATTTGGATGG TAACATTATT GGACCAAACT ATTGATAACG
 651   CGGACATTGT TAGACCAAAC TGAGAAGGAC ATGTCTGGAC TCCTGCTCCG
 701   TCTTCCATCA GCAGGTCGAT TCTTGTGGAA AATTAGCTCG AGGTGGCGCA
 751   CTATGTGAGG TAACTAGTAC TAAATTTTTC TTTGCTTAAT TTGTGCTATA
 801   TATACCTCAT CTAAATTATT GAATAGTCAC ACAAAGCAAA CATTTCTTGA
 851   TTTCTTCTCT ATCAACATAA CAAGTTTTGA TCATTTTTAG TGCAGAAATG
 901   GATTGTGTAA AACTTGTATT CCTTATGCTA TATACCTTTC TCTGTCAACT
 951   TGCTTTATCC TCATCCTTGC CTCATTGTG CCCCGAAGAT CAAGCTCTTT
1001   CTCTTCTACA ATTCAAGAAC ATGTTTACCA TTAATCCTAA TGCTTCTGAT
1051   TATTGTTACG ACATAAGAAC ATACGTAGAC ATTCAGTCAT ATCCAAGAAC
1101   TCTTTCTTGG AACAAAAGCA CAAGTTGCTG CTCATGGGAT GGCGTTCATT
1151   GTGACGAGAC GACAGGACAA GTGATTGCGC TTGACCTCCG TTGCAGCCAA
1201   CTTCAAGGCA AGTTTCATTC CAATAGTAGC CTCTTTCAAC TCTCCAATCT
1251   CAAAAGGCTT GATTTGTCTT TTAATAATTT CACTGGATCA CTCATTTCAC
1301   CAAAATTTGG TGAGTTTTCA AATTTGACGC ATCTCGATTT GTCGCATTCT
1351   AGTTTTACAG GTCTAATTCC TTCTGAAATC TGTCACCTTT CTAAACTACA
1401   CGTTCTTCGT ATATGTGATC AATATGGGCT TAGTCTTGTA CCTTACAATT
```

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FIGURE 2 CONTINUED

1451   TTGAACTGCT CCTTAAGAAC TTGACCCAAT TAAGAGAGCT CAACCTTGAA

1501   TCTGTAAACA TCTCTTCCAC TATTCCTTCA AATTTCTCTT CTCATTTAAC

1551   AACTCTACAA CTTTCAGGCA CAGAGTTACA TGGGATATTG CCCGAAAGAG

1601   TTTTTCACCT TTCCAACTTA CAATCCCTTC ATTTATCAGT CAATCCCCAG

1651   CTCACGGTTA GGTTTCCCAC AACCAAATGG AATAGCAGTG CATCACTCAT

1701   GACGTTATAC GTCGATAGTG TGAATATTGC TGATAGGATA CCTAAATCAT

1751   TTAGCCATCT AACTTCACTT CATGAGTTGT ACATGGGTCG TTGTAATCTG

1801   TCAGGGCCTA TTCCTAAACC TCTATGGAAT CTCACCAACA TAGTGTTTTT

1851   GCACCTTGGT GATAACCATC TTGAAGGACC AATTTCCCAT TTCACGATAT

1901   TTGAAAAGCT CAAGAGGTTA TCACTTGTAA ATAACAACTT TGATGGCGGA

1951   CTTGAGTTCT TATCCTTTAA CACCCAACTT GAACGGCTAG ATTTATCATC

2001   CAATTCCCTA ACTGGTCCAA TTCCATCCAA CATAAGCGGA CTTCAAAACC

2051   TAGAATGTCT CTACTTGTCA TCAAACCACT TGAATGGGAG TATACCTTCC

2101   TGGATATTCT CCCTTCCTTC ACTGGTTGAG TTAGACTTGA GCAATAACAC

2151   TTTCAGTGGA AAAATTCAAG AGTTCAAGTC CAAAACATTA AGTGCCGTTA

2201   CTCTAAAACA AAATAAGCTG AAAGGTCGTA TTCCGAATTC ACTCCTAAAC

2251   CAGAAGAACC TACAATTACT TCTCCTTTCA CACAATAATA TCAGTGGACA

2301   TATTTCTTCA GCTATCTGCA ATCTGAAAAC ATTGATATTG TTAGACTTGG

2351   GAAGTAATAA TTTGGAGGGA ACAATCCCAC AATGCGTGGT TGAGAGGAAC

2401   GAATACCTTT CGCATTTGGA TTTGAGCAAA AACAGACTTA GTGGGACAAT

2451   CAATACAACT TTTAGTGTTG GAAACATTTT AAGGGTCATT AGCTTGCACG

2501   GGAATAAGCT AACGGGGAAA GTCCCACGAT CTATGATCAA TTGCAAGTAT

2551   TTGACACTAC TTGATCTAGG TAACAATATG TTGAATGACA CATTTCCAAA

2601   CTGGTTGGGA TACCTATTTC AATTGAAGAT TTTAAGCTTG AGATCAAATA

2651   AGTTGCATGG TCCCATCAAA TCTTCAGGGA ATACAAACTT GTTTATGGGT

2701   CTTCAAATTC TTGATCTATC ATCTAATGGA TTTAGTGGGA ATTTACCCGA

2751   AAGAATTTTG GGGAATTTGC AAACCATGAA GGAAATTGAT GAGAGTACAG

2801   GATTCCCAGA GTATATTTCT GATCCATATG ATATTTATTA CAATTATTTG

2851   ACGACAATTT CTACAAAGGG ACAAGATTAT GATTCTGTTC GAATTTTGGA

2901   TTCTAACATG ATTATCAATC TCTCAAAGAA CAGATTTGAA GGTCATATTC

FIGURE 2 CONTINUED

```
2951  CAAGCATTAT  TGGAGATCTT  GTTGGACTTC  GTACGTTGAA  CTTGTCTCAC
3001  AATGTCTTGG  AAGGTCATAT  ACCGGCATCA  TTTCAAAATT  TATCAGTACT
3051  CGAATCTTTG  GATCTCTCAT  CTAATAAAAT  CAGCGGAGAA  ATTCCGCAGC
3101  AGCTTGCATC  CCTCACATTC  CTTGAAGTCT  TAAATCTCTC  TCACAATCAT
3151  CTTGTTGGAT  GCATCCCCAA  AGGAAAACAA  TTTGATTCGT  TCGGGAACAC
3201  TTCGTACCAA  GGGAATGATG  GGTTACGCGG  ATTTCCACTC  TCAAAACTTT
3251  GTGGTGGTGA  AGATCAAGTG  ACAACTCCAG  CTGAGCTAGA  TCAAGAAGAG
3301  GAGGAAGAAG  ATTCACCAAT  GATCAGTTGG  CAGGGGGTTC  TCGTGGGTTA
3351  CGGTTGTGGA  CTTGTTATTG  GACTGTCCGT  AATATACATA  ATGTGGTCAA
3401  CTCAATATCC  AGCATGGTTT  TCGAGGATGG  ATTTAAAGTT  GGAACACATA
3451  ATTACTACGA  AAATGAAAAA  GCACAAGAAA  AGATATTAGT  GAGTAGCTAT
3501  ACCTCCAGGT  ATTCCACTTG  ATCATTATCT  TTCAGAAGAT  TATTTTTTGT
3551  ATATCGATGA  AATTATCGAC  CTCCTTCATC  CTCAAAGCTC  TTAACTTTCA
3601  CTCTTCATTT  TTGAAAATTT  CAGGATTCAA  AGATTTCCGA  GTTCCCAGTT
3651  GCTTGGGATG  CAGATAAAAG  CCTTTTTATC  TTTCATAGTT  TCTTATCCTA
3701  TGAATAAAGA  TTTTATTTTC  ATTTGTCTAT  GGCACGTAGA  TATGTTCCGT
3751  CACTAAAAAC  ATTGTATTTC  TCTCAACTCT  TTCGTCACAT  GATATCAAAG
3801  AACACTTGAC  TTCAATTAAG  TTACTGTAGT  CTGCTATTTT  AATTTTTTCC
3851  ATTGAAACAC  AACTGACGTA  TCTTGAGAAA  GAGACTATGA  TCCCCCGGGC
3901  TGCAG
```

FIGURE 3

```
  1  MDCVKLVFLM  LYTFLCQLAL  SSSLPHLCPE  DQALSLLQFK  NMFTINPNAS
 51  DYCYDIRTYV  DIQSYPRTLS  WNKSTSCCSW  DGVHCDETTG  QVIALDLRCS
101  QLQGKFHSNS  SLFQLSNLKR  LDLSFNNFTG  SLISPKFGEF  SNLTHLDLSH
151  SSFTGLIPSE  ICHLSKLHVL  RICDQYGLSL  VPYNFELLLK  NLTQLRELNL
201  ESVNISSTIP  SNFSSHLTTL  QLSGTELHGI  LPERVFHLSN  LQSLHLSVNP
251  QLTVRFPTTK  WNSSASLMTL  YVDSVNIADR  IPKSFSHLTS  LHELYMGRCN
301  LSGPIPKPLW  NLTNIVFLHL  GDNHLEGPIS  HFTIFEKLKR  LSLVNNNFDG
351  GLEFLSFNTQ  LERLDLSSNS  LTGPIPSNIS  GLQNLECLYL  SSNHLNGSIP
401  SWIFSLPSLV  ELDLSNNTFS  GKIQEFKSKT  LSAVTLKQNK  LKGRIPNSLL
451  NQKNLQLLLL  SHNNISGHIS  SAICNLKTLI  LLDLGSNNLE  GTIPQCVVER
```

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FIGURE 3 CONTINUED

```
501    NEYLSHLDLS  KNRLSGTINT  TFSVGNILRV  ISLHGNKLTG  KVPRSMINCK

551    YLTLLDLGNN  MLNDTFPNWL  GYLFQLKILS  LRSNKLHGPI  KSSGNTNLFM

601    GLQILDLSSN  GFSGNLPERI  LGNLQTMKEI  DESTGFPEYI  SDPYDIYYNY

651    LTTISTKGQD  YDSVRILDSN  MIINLSKNRF  EGHIPSIIGD  LVGLRTLNLS

701    HNVLEGHIPA  SFQNLSVLES  LDLSSNKISG  EIPQQLASLT  FLEVLNLSHN

751    HLVGCIPKGK  QFDSFGNTSY  QGNDGLRGFP  LSKLCGGEDQ  VTTPAELDQE

801    EEEEDSPMIS  WQGVLVGYGC  GLVIGLSVIY  IMWSTQYPAW  FSRMDLKLEH

851    IITTKMKKHK  KRY
```

FIGURE 4

```
1      CATTTCTTGA  TTTCTTCTCT  ATCAACATAA  CAAGTTTTGA  TCATTTTTAG

51     TGCAGAAATG  GATTGTGTAA  AACTTGTATT  CCTTATGCTA  TATACCTTTC

101    TCTGTCAACT  TGCTTTATCC  TCATCCTTGC  CTCATTTGTG  CCCCGAAGAT

151    CAAGCTCTTT  CTCTTCTACA  ATTCAAGAAC  ATGTTTACCA  TTAATCCTAA

201    TGCTTCTGAT  TATTGTTACG  ACATAAGAAC  ATACGTAGAC  ATTCAGTCAT

251    ATCCAAGAAC  TCTTTCTTGG  AACAAAGCA   CAAGTTGCTG  CTCATGGGAT

301    GGCGTTCATT  GTGACGAGAC  GACAGGACAA  GTGATTGCGC  TTGACCTCCG

351    TTGCAGCCAA  CTTCAAGGCA  AGTTTCATTC  CAATAGTAGC  CTCTTTCAAC

401    TCTCCAATCT  CAAAAGGCTT  GATTTGTCTT  TTAATAATTT  CACTGGATCA

451    CTCATTTCAC  CAAAATTTGG  TGAGTTTTCA  AATTTGACGC  ATCTCGATTT

501    GTCGCATTCT  AGTTTTACAG  GTCTAATTCC  TTCTGAAATC  TGTCACCTTT

551    CTAAACTACA  CGTTCTTCGT  ATATGTGATC  AATATGGGCT  TAGTCTTGTA

601    CCTTACAATT  TTGAACTGCT  CCTTAAGAAC  TTGACCCAAT  TAAGAGAGCT

651    CAACCTTGAA  TCTGTAAACA  TCTCTTCCAC  TATTCCTTCA  AATTTCTCTT

701    CTCATTTAAC  AACTCTACAA  CTTTCAGGCA  CAGAGTTACA  TGGGATATTG

751    CCCGAAAGAG  TTTTTCACCT  TTCCAACTTA  CAATCCCTTC  ATTTATCAGT

801    CAATCCCCAG  CTCACGGTTA  GGTTTCCCAC  AACCAAATGG  AATAGCAGTG

851    CATCACTCAT  GACGTTATAC  GTCGATAGTG  TGAATATTGC  TGATAGGATA

901    CCTAAATCAT  TTAGCCATCT  AACTTCACTT  CATGAGTTGT  ACATGGGTCG

951    TTGTAATCTG  TCAGGGCCTA  TTCCTAAACC  TCTATGGAAT  CTCACCAACA
```
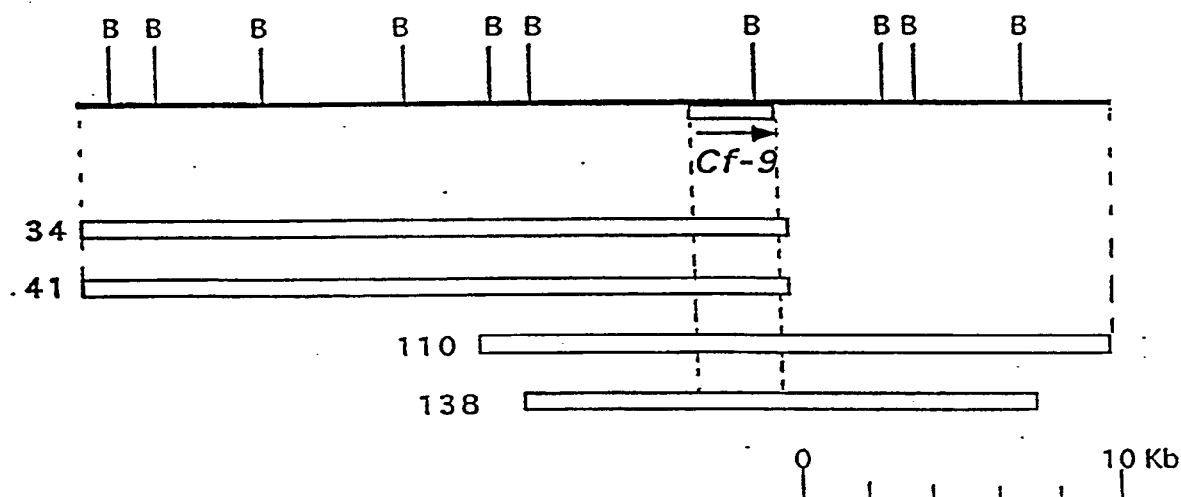
FIGURE 4 CONTINUED

```
1001  TAGTGTTTTT GCACCTTGGT GATAACCATC TTGAAGGACC AATTTCCCAT

1051  TTCACGATAT TTGAAAAGCT CAAGAGGTTA TCACTTGTAA ATAACAACTT

1101  TGATGGCGGA CTTGAGTTCT TATCCTTTAA CACCCAACTT GAACGGCTAG

1151  ATTTATCATC CAATTCCCTA ACTGGTCCAA TTCCATCCAA CATAAGCGGA

1201  CTTCAAAACC TAGAATGTCT CTACTTGTCA TCAAACCACT TGAATGGGAG

1251  TATACCTTCC TGGATATTCT CCCTTCCTTC ACTGGTTGAG TTAGACTTGA

1301  GCAATAACAC TTTCAGTGGA AAAATTCAAG AGTTCAAGTC CAAAACATTA

1351  AGTGCCGTTA CTCTAAAACA AAATAAGCTG AAAGGTCGTA TTCCGAATTC

1401  ACTCCTAAAC CAGAAGAACC TACAATTACT TCTCCTTTCA CACAATAATA

1451  TCAGTGGACA TATTTCTTCA GCTATCTGCA ATCTGAAAAC ATTGATATTG

1501  TTAGACTTGG GAAGTAATAA TTTGGAGGGA ACAATCCCAC AATGCGTGGT

1551  TGAGAGGAAC GAATACCTTT CGCATTTGGA TTTGAGCAAA AACAGACTTA

1601  GTGGGACAAT CAATACAACT TTTAGTGTTG GAAACATTTT AAGGGTCATT

1651  AGCTTGCACG GGAATAAGCT AACGGGGAAA GTCCCACGAT CTATGATCAA

1701  TTGCAAGTAT TTGACACTAC TTGATCTAGG TAACAATATG TTGAATGACA

1751  CATTTCCAAA CTGGTTGGGA TACCTATTTC AATTGAAGAT TTTAAGCTTG

1801  AGATCAAATA AGTTGCATGG TCCCATCAAA TCTTCAGGGA ATACAAACTT

1851  GTTTATGGGT CTTCAAATTC TTGATCTATC ATCTAATGGA TTTAGTGGGA

1901  ATTTACCCGA AAGAATTTTG GGGAATTTGC AAACCATGAA GGAAATTGAT

1951  GAGAGTACAG GATTCCCAGA GTATATTTCT GATCCATATG ATATTTATTA

2001  CAATTATTTG ACGACAATTT CTACAAAGGG ACAAGATTAT GATTCTGTTC

2051  GAATTTTGGA TTCTAACATG ATTATCAATC TCTCAAAGAA CAGATTTGAA

2101  GGTCATATTC CAAGCATTAT TGGAGATCTT GTTGGACTTC GTACGTTGAA

2151  CTTGTCTCAC AATGTCTTGG AAGGTCATAT ACCGGCATCA TTTCAAAATT

2201  TATCAGTACT CGAATCTTTG GATCTCTCAT CTAATAAAAT CAGCGGAGAA

2251  ATTCCGCAGC AGCTTGCATC CCTCACATTC CTTGAAGTCT TAAATCTCTC

2301  TCACAATCAT CTTGTTGGAT GCATCCCCAA AGGAAAACAA TTTGATTCGT

2351  TCGGGAACAC TTCGTACCAA GGGAATGATG GGTTACGCGG ATTTCCACTC

2401  TCAAAACTTT GTGGTGGTGA AGATCAAGTG ACAACTCCAG CTGAGCTAGA
```

FIGURE 4 CONTINUED

```
2451  TCAAGAAGAG  GAGGAAGAAG  ATTCACCAAT  GATCAGTTGG  CAGGGGGTTC

2501  TCGTGGGTTA  CGGTTGTGGA  CTTGTTATTG  GACTGTCCGT  AATATACATA

2551  ATGTGGTCAA  CTCAATATCC  AGCATGGTTT  TCGAGGATGG  ATTTAAAGTT

2601  GGAACACATA  ATTACTACGA  AAATGAAAAA  GCACAAGAAA  AGATATTAGT

2651  GAGTAGCTAT  ACCTCCAGGA  TTCAAAGATT  TCCGAGTTCC  CAGTTGCTTG

2701  GGATGCAGAT  AAAAGCCTTT  TTATCTTTCA  TAGTTTCTTA  TCCTATGAAT

2751  AAAGATTTTA  TTTTCATTTG  TCTATGGCAC  GTAGATATGT  TCCGTCACTA

2801  AAAACATTGT  ATTTCTCTCA  ACTCTTTCGT  CACATGATAT  CAAAGAACAC

2851  TTGACTTCAA  TTAAGTTAAA  AAAAAAAAAA
```

FIGURE 5

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/29   C12N15/82   C12N1/21   C12N5/10   A01H5/00
         A01H5/10   C12Q1/68   A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A01H   A01N   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT JOURNAL, vol.2, no.3, 1992 pages 367 - 373 TOUBART, P., ET AL. 'Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of Phaseolus vulgaris L.' see the whole document ---	1
X	PLANT PHYSIOLOGY, vol.102, May 1993 pages 133 - 138 STOTZ, H.U., ET AL. 'Molecular characterization of a polygalacturonase inhibitor from Pyrus communis L. cv Bartlett' see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1995	3 0. 05. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Inte. .ional Application No

PCT/GB 94/02812

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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P,X	SCIENCE, vol.266, 4 November 1994, LANCASTER, PA US pages 789 - 793 JONES, D.A., ET AL. 'Isolation of the tomato Cf-9 gene for resistance to Cladosporium fulvum by transposon tagging' see the whole document ---	1-3,6,7
A	MOL. PLANT-MICROBE INTERACT., vol.6, 1993 pages 348 - 357 JONES, D.A., ET AL. 'Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2,Cf-4,Cf-5, and Cf-9 genes for resistance to Cladosporium-fulvum' see the whole document ---	1-15
A	CURRENT PLANT SCIENCE AND BIOTECHNOLOGY IN AGRICULTURE, VOL. 10. ADVANCES IN MOLECULAR GENETICS OF PLANT-MICROBE INTERACTIONS, VOL. 1; 5TH INTERNATIONAL SYMPOSIUM ON THE MOLECULAR GENETICS OF PLANT-MICROBE INTERACTIONS, INTERLAKEN, SWITZERLAND., 1991, SEPT. 9-14, 1990. pages 276 - 279 DICKINSON, M., ET AL. 'Strategies for the cloning of genes in tomato for resistance to Fulvia-fulvia' see the whole document ---	1-15
A	GENET. ENG. (N.Y.), vol.14, 1992 pages 99 - 124 BENNETZEN, J.L., ET AL. 'Approaches and progress in the molecular cloning of plant disease resistance genes' see the whole document ---	1-15
A	WO,A,91 15585 (RIJKSLANDBOUWUNIVERSITEIT WAGENINGEN) 17 October 1991 see the whole document ---	1-15
A	SCIENCE, vol.262, 26 November 1993, LANCASTER, PA US pages 1432 - 1436 MARTIN, G.B., ET AL. 'Map-based cloning of a protein kinase gene conferring disease resistance in tomato' see the whole document ---	1-15

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page 2 of 3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol.258, 5 November 1992 pages 985 - 987 JOHAL, G.S., ET AL. 'Reductase activity encoded by the HM1 disease resistance gene in maize' see the whole document -----	1-15

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INTERNATIONAL SEARCH REPORT

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		CA-A-	2125045	10-06-93
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		AU-B-	642252	14-10-93
		AU-A-	7684591	30-10-91
		EP-A-	0474857	18-03-92
		JP-T-	5505110	05-08-93